REMARKS

Applicants submit this response as a supplement to the earlier filed response of April 26, 2004 which replied to the office action mailed on February 18, 2004 in the above-identified application.

In this supplemental response applicants have amended claims 1 and 10 to delete the term "perennial" which was added in response to the lack of enablement rejection under 35 U.S.C. 112, first paragraph. Applicants believe that this limitation is unnecessary and would unduly restrict the claims, particularly since applicants have exemplified plants such as *Nicotiana glauca* which are not perennials (see page 11 paragraph [0040], and pages 13-15 [0047] to [0053].) Furthermore, applicants submit that it is well recognized within the plant pathology art that *N. glauca* is a suitable indicator plant for crown gall assays. (See *Annual Review of Phytopathology*, September (1999), vol. 37, pp. 53-80, specifically the section on "Genes Associated With Host-Pathogen Interactions".) A copy of this reference is enclosed herewith. Applicants note that the operative idea in the experiments described in the present application is that galling on *N. glauca* is representative of galling of plants in general, because *N. glauca* is well known as an indicator plant.

This belief that crown gall can be controlled in all plant species susceptible to the disease is further supported by the additional grapevine experimental data and photographic exhibits submitted with the response filed on April 26, 2004. The ability for the method of the invention to control crown gall in grapes demonstrates that indeed taxonomically divergent species of plants have been found that are responsive to the crown gall controlling α-proteobacteria. The results also demonstrate the efficacy of using the methods and compositions of the invention to control crown gall beyond simply *Nicotiana glauca*. Therefore, biocontrol of galling by a TFX-producing strain is indicative of biocontrol on a plant species susceptible to crown gall disease.

Applicants also submit that Claims 1, 10 and 19 have been amended herein to replace the term "forming" with the term "controlling" which was inadvertently included in the response filed on April 26, 2004.

Furthermore, Applicants submit that Claims 4, 13 and 22 have been currently amended to include the term "pT2TFXK" outside of any parentheticals simply for clarification purposes. It is noted that the *Agrobacterium vitis* F2/5 bearing the pT2TFXK plasmid was deposited with the ATCC as Patent Deposit Designation PTA-2356 (see

International ATCC form submitted to the USPTO along with Applicants earlier response on April 26, 2004). No new matter has been added with the introduction of the claim amendments provided herein.

Additionally and alternatively to the fact that crown gall can be controlled in all plant species susceptible to the disease, applicants respectfully direct the Examiner's attention to *In re Goodman*, 11 F.3d 1046, 1052, 29 USPQ2d 2010 (Fed. Cir. 1993). The Federal Circuit in *In re Goodman* addressed several different patent related issues, in doing so it also acknowledged a basic scientific fact determinative of the level of enablement required to support the claims. The Court in its background discussion referred to the USPTO Board of Appeals conclusions and acknowledged that it is a well known in the art that bacterium *Agrobacterium tumefaciens* can infect dicotyledonous plants by attaching to the plant cell wall and introducing a particular piece of its Ti plasmid DNA into the plant cell. The Court also noted that the Ti plasmid is a T umor i nducing plasmid found in the bacterium Agrobacterium tumefaciens that is responsible for crown gall disease in plants (producing tumors). The Court reiterated that Board's conclusions and stated that the Agrobacterium-mediated transformation method of plant transformation works with all dicotyledonous plant cells.

As such, Applicants submit that by limiting the plants of the invention to "plant species susceptible to crown gall disease" applicants have inherently limited the plants to dicots because as recited in *In re Goodman,* Agrobacterium, an α-proteobacteria, only affect dicotyledonous plant cells. Since dicotyledonous plants would inherently be included in plants susceptible to crown gall disease, it logically follows that the disease in dicots can be controlled by genetically engineering α-proteobacteria to produce trifolitoxin. In other words only plants that are susceptible to crown gall disease can be cured by the methods of the invention (i.e., α-proteobacteria producing trifolitoxin).

Lastly, applicants respectfully acknowledge that Claims 2-4, 8, 11-13, 17, and 20-22 are deemed free from prior art. However, applicants would like to clarify that the reason for such a determination should not be because the prior art does not teach Agrobacterium or Agrobacterium *vitis* F2/5 as bacterial host for trifolitoxin production, but rather because the prior art does not teach crown gall biocontrol with a TFX-producing strain.

Therefore, applicants respectfully request that in view of the claim amendments and the accompanying remarks presented herein, the above- identified rejection should be reconsidered and withdrawn. Also, applicants would appreciate receiving a timely Notice of Allowance in this case.

No fee is believed to be due in connection with this Supplemental Response. However, if any fee is due in this or any subsequent response, please charge the fee to the same Deposit Account No. 17-0055.

Respectfully submitted,

Sara D. Vinarov
Reg. No.: 48,524
Attorney for Applicants
QUARLES & BRADY LLP
P.O. Box 2113
Madison, WI 53701

TEL 608/251-5000
FAX 608/251-9166

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CONTENTS

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CROWN GALL OF GRAPE: Biology and Disease Management

Thomas J. Burr and Leon Otten
Department of Plant Pathology, New York State Agricultural Experiment Station, Cornell University, Geneva, NY 14456 and Institute of Plant Molecular Biology, 12 rue du General Zimmer, 67084 Strasbourg, France; e-mail: tjb1@cornell.edu

Key Words *Agrobacterium vitis*, evolution, genetic diversity, plasmids, taxonomy

■ **Abstract** Not until 1973 was it reported that strains of Agrobacterium that cause crown gall disease of grape form a specific group (later characterized as *Agrobacterium vitis*). Tumorigenic and nontumorigenic *A. vitis* have since been isolated from infected and symptomless grapes worldwide. Research on the genetic makeup of *A. vitis* has led to an improved understanding of pathogen biology and bacterial evolution. In addition, the identification of significant gene sequences has facilitated the development of PCR and RFLP-based identification procedures that continue to improve the detection of *A. vitis* in plants and soil. Current control practices rely on the use of disease-resistant cultivars, cultural practices that minimize plant injury, and the production of pathogen-free vines. Promising future controls include employment of biological control agents and development of crown gall–resistant transgenic grapevines.

INTRODUCTION

Crown gall has been recognized as a plant disease of worldwide importance on many plant species for more than 100 years (28). A discovery by Braun & Mandle (9) in 1948 provided major insight into the crown gall infection process and paved the way for intensive research on the molecular biology of infection: Once plants were infected by *Agrobacterium tumefaciens*, the tumor tissue could be cultured on a defined, hormone-free medium in the absence of the bacterium. Thus, something of unknown nature [the tumor-inducing factor (TIP)] was being transferred by the bacterium to the plant cell. Almost 30 years later, it was demonstrated that the genes associated with tumor formation are carried on a plasmid (Ti plasmid). Major discoveries included the following: *A. tumefaciens* transfers tumorigenicity factors from tumorigenic to nontumorigenic strains (52); the presence of the Ti plasmid in the bacterium is positively correlated with tumorigenicity (111); and bacterial DNA from the Ti plasmid (the transfer of T-DNA) is integrated into the plant genome (29). Ti plasmids also carry genes that are involved in synthesis and catabolism of a diverse group of small molecules called opines. Opines are synthesized in

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and secreted from galls. The rather unique ability of Agrobacterium to catabolize them gives the bacterium a competitive advantage in the vaccinity of galls. Today, highly active research programs continue to study the mechanisms by which Agrobacterium transfers Ti plasmids between bacteria and T-DNA into plants.

Some of the earliest reports of crown gall were of the disease on grape (41). It was repeatedly observed that injuries on grape trunks caused by freezing temperatures provide common sites for crown gall infections and that grape cultivars vary in their susceptibility to the disease. The widespread occurrence of the disease and variation in cultivar susceptibility are reflected in results of a multicountry crown gall survey reported by the Office International de la Vigne et du Vin, which was recently summarized (14). The impact of crown gall on vine growth and yield was assessed in a four-year study in California on the cultivar Zinfandel (86). Vines with 50% or greater of their trunk circumference affected with crown gall had significantly less growth than mildly diseased vines had. Crown gall may also cause significant losses in nurseries by affecting vine growth and necessitating the destruction of infected plants.

AGROBACTERIUM VITIS

Although tumorigenic *Agrobacterium* spp. were isolated from grape galls for many years, strains from grape were first compared with those from other plants by Panagopoulos & Psallidas in 1973 (72). By comparing responses to various biochemical and physiological tests, they concluded that strains from grape formed a distinct group and that their tumorigenicity was often host-limited. Kerr & Panagopoulos (53) and Süle (92) later proposed that strains from grape be classified as biovar 3 (or biotype 3) of *A. tumefaciens*. In the following years, it became apparent that the vast majority of strains isolated from crown gall–diseased grapevines worldwide belonged to biovar 3 and that there are wide host range (WHR) and limited host range (LHR) strains. In addition to having their physiological and biochemical traits, biovar 3 strains were insensitive to agrocin 84 in vitro, and their tumorigenicity on grape was not suppressed by *A. radiobacter* strain K84. Biovar 3 strains were also differentiated from other biovars by reaction to a monoclonal antibody (4) and by their ability to induce a grape-specific necrosis (16).

Further taxonomic evaluation of biovar 3 strains by Ophel & Kerr in 1990 resulted in the naming of new species, *A. vitis* (62). In addition to the criteria listed above, they compared DNA homology between *A. vitis* strains and other *Agrobacterium* spp. More recently, several other methods have been used to characterize *A. vitis*. Jarvis et al (48) used fatty acid analysis to identify species of 65 strains of *Agrobacterium* spp. and 150 strains of *Rhizobium* and *Sinorhizobium*. A two-dimensional plot analysis of fatty acid compositions revealed that *A. vitis* is more similar to *Rhizobium galegae* than it is to other *A. tumefaciens* or *A. rhizogenes*. Bouzar & Jones (8) also showed that *A. vitis* can be differentiated from other *Agrobacterium* spp. by fatty acid analysis. It was determined that *cis*-vaccenic acid is predominant in all Agrobacterium strains, whereas the level of 3-hydroxypalmitic acid differs among species: *A. vitis* produces significantly less than *A. tumefaciens* or *A. rhizogenes*. Interestingly, *A. vitis* produces the highest detectable level of 10 methylnonadecenoic acid, and only *A. vitis* and *A. rhizogenes* produce detectable levels of arachidonic acid.

A. vitis was also differentiated from other *Agrobacterium* spp. by using the GN Microplate system (BioLog, Inc.). They form a distinct group based on utilization of 95 different carbon sources. Major differences between *A. vitis* and all other species tested were that relatively few *A. vitis* strains utilized D-galactonic acid lactone, whereas the majority of strains utilized *p*-hydroxyphenylacetic acid. With BioLog and fatty acid analysis, species of Agrobacterium formed distinct clusters regardless of tumorigenicity.

In addition to fatty acid analysis, Weibgen et al (107) investigated the lipopolysaccharide (LPS) composition of nine Agrobacterium strains (including three *A. vitis* strains) and determined that *A. vitis* and *A rubi* contain an R-type LPS, whereas *A. tumefaciens* and *A. rhizogenes* have very long O-chains with more than 20 O-repeating subunits. The rare sugar derivative 3-deoxy-*lyxo*-heptulosaric acid was found at trace amounts in all species but at highest levels in all LPS fractions of *A. vitis*. An *A. vitis*–specific monoclonal antibody did not react with the isolated LPS from the bacterium.

Comparisons of *A. vitis* with other related species were also made at the DNA level. Sawada compared DNA sequences of a short variable region (nucleotides 1019–1200) of the 16S rRNA gene (84). In this analysis, *A. vitis* was found to be most similar to *A. rubi* (only 2 nucleotide differences) and most dissimilar to *A. tumefaciens* (17 nucleotide differences). In a related study, 16S rRNA sequences from several genera in Rhizobiaceae were compared (108). The single *A. vitis* strain analyzed was 94–96% similar to *A. rhizogenes*, *A. rubi*, and *A. tumefaciens* but even more similar to a strain of *Rhizobium galegae* (same conclusion as for fatty acid analysis discussed above). More detailed and informative comparisons of entire *A. vitis* rRNA sequences are discussed later in this review.

Irelan & Meredith, using RAPDs, detected a high degree of inter- and intraspecific genetic diversity in total genomic DNA from a collection of Agrobacterium strains that included *A. vitis* (46). Four random primers were found to be most effective for detecting diversity, and *A. vitis* strains (all from California) were more diverse than other species, with only 12% of the PCR products being shared between strains. It was significant that the different species of *Agrobacterium* clustered separately according to RAPD patterns.

GENETIC DIVERSITY

The genetic diversity of *A. vitis* has been studied more thoroughly than for other *Agrobacterium* spp., probably because grape is a high-value crop and significant outbreaks of crown gall occurred in the United States and Europe after unusually

cold winters in the mid-1980s. Because of its phytopathological significance, many strains are available from various grape-growing areas in the world including Germany, France, Australia, Hungary, South Africa, and the United States. As is the case for *A. tumefaciens* and *A. rhizogenes*, most known pathogenic properties of *A. vitis* are encoded by genes on the Ti plasmid. Regions of the *A. vitis* genome, particularly the Ti plasmids, have been studied extensively. They show considerable diversity, which makes it possible to define genetic relationships between various strains, to identify representatives of the different groups, and to establish the phytopathological properties of model strains. It has also been possible to develop efficient identification and characterization methods for large numbers of strains.

Ti Plasmid Structure

Restriction maps, cloned fragments, and sequences are now available for the major types of *A. vitis* Ti plasmids. References to sequences of *A. vitis* strains (now adding up to about 110 kb) are listed in Table 1. The following Ti plasmids have been cloned and mapped:

1. Three O/C type Ti plasmids that have large TA-regions (O/C refers to genes that encode the synthesis of the opines octopine and cucumopine. TA refers to one of the two T-DNAs on the plasmids): pTiTm4 (256 kb) (69), pTiHm1 (258 kb) (104), and pTi2608 (248 kb) (37).
2. Two O/C type Ti plasmids that have small TA-regions: pTiAB3 (234 kb) (104) and pTiAg57 (224 kb) (104).
3. A nopaline type Ti plasmid, pTiAB4 of 157 kb (65), that is highly conserved in different *A. vitis* nopaline strains.

O/C large TA, O/C small TA, and nopaline type plasmids have identical virulence regions (65, 104) but differ in other regions. The ancestral Ti plasmid type has not yet been determined but the characteristic mixture of common DNA and specific DNA in the plasmids is a strong indication for plasmid recombination. Interestingly, the nucleotide sequences of common regions are more than 99.9% homologous, which suggests that the evolution of plasmids proceeds mainly by large-scale events like insertions and deletions (65, 71, 104). In one case, it was shown that a direct 2.3-kb repeat present in various O/C Ti plasmids can lead to the loss of a 66-kb fragment containing the TB-region (37). Large-scale events like deletions or insertions complicate attempts to construct phylogenetic trees. For example, we have shown that the genetic relationships between the Ti plasmids cannot be analyzed with RFLP-based techniques but only by careful analysis of Ti plasmid maps (104). In addition, repeated clonal expansion of only a few Ti types has led to a lack of evolutionary intermediates, which makes it even more difficult to reconstruct the evolutionary history of these structures. Such reconstructions are not only interesting from a theoretical point of view but are important for

TABLE 1 DNA that has been sequenced from *Agrobacterium vitis* as of December 1998

Strain (Ti type)	Description	Size (nt)	Accession no.
Chromosome			
K305 (O/C)	Repeated sequence, partial	132	S68163
CG49 (N)	*pehA*	2866	U73161
K309 (O/C)	*rrnA* operon	7276	U45329
S4 (V)	*rrnA* operon	7534	U28505
Ti plasmid			
CG474 (O)	Complete T-DNA	14960	U83986
AB4 (N)	*iaaM* to *nos* gene	6482	X77327
Tm4 (O/C)	Complete T-DNA	15463	U83987
Ag162 (O/C)	*iaaH*	1835	AF039169
AB3 (O/C)	IS*869*	863	X53945
AB3 (O/C)	IS*868*	1362	X55075
AB3 (O/C)	TA, *ocs*, partial	200	M63058
AB3 (O/C)	TA, right border	307	M63056
AB3 (O/C)	*acs*, partial	424	M91188
Ag57 (O/C)	TA, right border	185	M63057
Ag57 (O/C)	IS*870*	1754	Z18270
2608 (O/C)	RSAv-1 repeat	2563	Z22732
Ag162 (O/C)	*virA*	2730	X05241
Ag57 (O/C)	*virF*	922	AF044200
S4 (V)	T1-DNA	3720	M91608
S4 (V)	T2-DNA	5516	M91609
S4 (V)	T3-DNA	3271	M91610
TAR region			
AB4 (N)	*ttuA-ttuE*	7163	U25634
AB4 (N)	*ttuC'*	1523	AF010261
AB4 (N)	ORFZ2	693	AF010414
pTiAB3 (O/C)	*ttuA*	227	AF010413
pTiAB3 (O/C)	*ttuC*	3405	AF010262
pTiAB3 (O/C)	*ttuC'*	3172	AF010263
pTiAB3 (O/C)	ORFZ2	1041	AF010415
pTiAB3 (O/C)	*ttuA*-ORFZ2	11928	U32375

establishing the relatedness of the different plasmid forms and predicting their oncogenic properties and for predicting their capacity to evolve.

4. Many regions of the Ti plasmid from the vitopine strain S4 (262 kb) (39) are homologous to fragments of other Ti plasmids, but its origin of replication belongs to a new incompatibility group called IncRh-4 (96), and its virulence genes are arranged differently from other Ti plasmids.

Chromosome Structure

As mentioned previously, strains from grape were grouped together as biovar 3 strains of *A. tumefaciens* until further molecular and classical microbiological analysis led to the naming of a separate species, *A. vitis*. These studies have been confirmed and extended by sequence analysis of ribosomal RNA sequences. *A. vitis* strains contain four ribosomal RNA operons, *rrnA–D* (66, 67). The *Agrobacterium rrn* operons are about 7 kb in size and show conserved and variable regions. The variable regions can be used to construct very detailed intraspecific phylogenetic trees, whereas the conserved regions can be used to define relationships at higher taxonomic levels, for example, in comparisons with other genera like *Rhizobium* and *Bradyrhizobium*. Sequence studies on the *rrn* genes from the Rhizobiaceae family have shown that agrobacteria and rhizobia are not well-defined groups. *A. vitis*, for example, is more closely related to *R. galegae* than to other *Agrobacterium* species, whereas *A. rhizogenes* is more closely related to *Rhizobium tropici* than it is to other agrobacteria (109). These conclusions, however, require further support from comparisons of other chromosomal sequences (63).

The *rrnA* operons of two representative *A. vitis* strains (K309, with an O/C, large TA Ti plasmid, and strain S4, with a vitopine Ti plasmid) have been completely sequenced and are the first such sequences for *Agrobacterium* spp. (66, 67). K309 is the *A. vitis*–type strain and is maintained as NCPPB3554. It should be stressed that it is important in *rrn* studies to compare orthologous copies, a requirement seldom taken into account when ribosomal sequences are used to construct phylogenetic trees. Since ribosomal RNAs are considered to be highly conserved within a species, it came as a surprise that the so-called B8 stem-loop structure at the 5′ end of the 23S rRNA of K309 was 142 nt shorter than that of S4. Such features can be used as synapomorphic markers to allow cladistic analysis within and between strains. The 16S–23S intergenic region (IGS) contains highly conserved and more variable regions. Homologies for the sequences of the two *A. vitis* strains vary from 56.7% for the 5′ end of the *rrn* operon to 100% for the three tRNA genes and the 5S gene. About 325 positions within the *rrn* operons of K309 and S4 differ. In the regions of secondary structure, compensatory base-pair changes are found, which confirms the stem-loop models for the RNAs (67). Because of the nonrandom pattern of homology distribution at the *rrn* level, it is important to use the same *rrn* regions (preferably including slowly and rapidly evolving fragments) when results of different studies are compared.

It would be interesting to include the three other *rrn* copies of the *A. vitis* genome in such studies. *rrn* operon evolution involves amplification and possibly gene conversion (modification of a copy by unilateral sequence transfer from another copy), a phenomenon that must be taken into account when establishing phylogenetic relationships from such sequences. Evidence for gene conversion in *A. vitis* has been obtained in at least two cases: for the AB4 *ipt* gene (65) and for different *ttuC* genes (81).

The *A. vitis* ribosomal sequences now available have allowed a detailed comparison of a number of strains by using IGS primers. These studies (61, 68) have shown the potential of this type of analysis to detect small differences between closely related strains, to rapidly classify strains, and to identify new strain types. It was also shown that restriction enzyme analysis of total bacterial DNA (using standard strain patterns as markers) can be used for preliminary identification. Evidence for the existence of diverse chromosomal types in *A. vitis* was obtained from these studies. It was possible to separate tumorigenic *A. vitis* into distinct groups (and sometimes to the strain level) based on DNA fingerprints of an intergenic spacer region (the region between the 16S and 23S rRNA genes) and of the 5′ end of the 23S gene. Although more diversity among strains was detected by RAPD analysis, this method also resulted in strain groupings that were highly correlated with those identified by the rRNA gene fingerprinting. It was determined that well-defined groups exist within the species and that chromosomal type is highly correlated with the type of Ti plasmid carried by strains. For example, 25 of 26 strains (originating from the United States and Hungary) carrying nopaline type Ti plasmids had identical IGS and 5′ 23S fingerprints. Strains carrying vitopine plasmids were divided into three clusters, and those with O/C plasmids formed clusters with large or small TA-regions. Note that PCR-RFLP analysis may not distinguish between point mutations and large-scale events like insertions or deletions of longer DNA stretches; however, these events should be considered separately when a phylogenetic tree is constructed. Further studies are therefore needed to interpret PCR-RFLP patterns in terms of evolutionary change within the *A. vitis* group; these could yield quantitative data on degree of diversity and speed of evolution.

As mentioned previously, only nontumorigenic strains of *A. vitis* were isolated from feral *V. riparia*. When 24 strains were analyzed by IGS fingerprinting, they were highly diverse and generated 13 different fingerprints, none identical to fingerprints of tumorigenic strains (22). It will be interesting to compare in more detail the genomes of tumorigenic and nontumorigenic strains. Such research may lead to a better understanding of factors that influence the apparent Ti plasmid/chromosome specificity and further elucidate the evolutionary development of *A. vitis* as a grape pathogen.

GENES ASSOCIATED WITH HOST-PATHOGEN INTERACTIONS

A. vitis shows a high degree of host specificity for grape. Although other *Agrobacterium* spp. have been identified in grape rhizospheres (17), *A. vitis* is by far the predominant species detected in grape crown galls. Genes that are associated with

host interactions are known to reside on Ti plasmids, on plasmids that encode tartrate utilization, and on the chromosome.

Ti Plasmid Genes

Early molecular studies on *A. vitis* concentrated mainly on the host range properties of two strains of the octopine type (later determined to be O/C types): Ag162 and Ag57 (12, 13, 43, 55, 101, 110). Ag162 and Ag57 differ in host range from the *A. tumefaciens* strain A6 that carries an octopine-type Ti plasmid. Whereas A6 has a wide host range (WHR) that allows tumor induction on common test plants such as *Nicotiana tabacum* or *Datura stramonium*, Ag162 and Ag57 expressed a limited host range (LHR), inducing tumors on *Vitis vinifera* and only a few test plants such as *Lycopersicon esculentum* and *Kalanchoe tubiflora*. Three host range–related genes on the Ti plasmid were identified: *virA*, *virC*, and the T-DNA oncogene *ipt*, which encodes the synthesis of a cytokinin.

Vir *Genes* The virulence system of *A. tumefaciens* has been studied extensively. It comprises a large number of genes located in a compact 30-kb virulence region [for reviews see (50, 88)]. These genes are generally responsible for encoding proteins that liberate the T-DNA from the Ti plasmid and transport it to the plant cells. The *virA* gene encodes a sensory protein that detects the presence of monosaccharides and phenolic compounds such as acetosyringone secreted by wounded plant cells (27). In the presence of these signal molecules, VirA undergoes autophosphorylation and transfers a phosphate group to VirG, which acts as a transcriptional regulator for other *vir* genes (49).

Studies on *virA* of pTiAg162 showed that this gene is partially responsible for the limited host range of *A. vitis* Ag162. Replacement of its *virA* gene with the A6 *virA* enhanced host range (110). The LHR VirA protein has only 45% amino acid homology to the WHR VirA. Further studies demonstrated that domains acting as receptors for the sugar and phenolic signals are conserved in the LHR VirA protein. The LHR *virA* differs from WHR *virA* in that its promoter region is not inducible by acetosyringone (103).

The *virC* gene of WHR strains prevents tumor induction on grapevine by the induction of a hypersensitive response, and *virC*-minus mutants are virulent (110). Although the precise mechanism for the hypersensitive response has not been established, the WHR *virC* gene likely increases the efficiency of T-DNA transfer. On grape this may lead to toxic levels of hormones encoded by the WHR T-DNA oncogenes.

The *virD2* gene encodes an endonuclease that recognizes the T-DNA borders and liberates the T strand, a single-stranded DNA molecule that is subsequently exported to the plant cell. The T strand is protected by the *virE*-encoded single-stranded DNA-binding protein VirE2, which contains a nuclear targeting signal. Transgenic plants producing VirE2 can complement a *virE* mutant, which shows that VirE2 acts in the plant cell probably by protecting and transporting the T strand. A similar complementation has been achieved for the *virF* gene. This gene is found in octopine strains of *A. tumefaciens* but is lacking in nopaline strains and its precise function is unknown. Recently, *virF* was found in O/C and nopaline strains of *A. vitis* at an unusual position within the *vir* region. The *A. vitis* and *A. tumefaciens virF* genes are highly similar; both contain a *vir* box (conserved region of *vir* genes required for signal-regulated transcription) and are induced by acetosyringone (85).

Finally, the large *virB* operon of *A. tumefaciens* contains 11 open reading frames. A number of these encode proteins that assemble into a sex-pilus and a periplasmic membrane-located transfer complex (50, 88). The *virB* genes of *A. vitis* have not yet been studied.

T-DNA Oncogenes The main tumor-inducing genes of octopine and nopaline strains of *Agrobacterium tumefaciens* are the *ipt* and *iaa* genes. The *ipt* gene encodes an isopentenyl transferase that catalyzes the synthesis of cytokinins. The *iaa* genes encode indoleacetic acid (IAA) synthesis in two steps: *iaaM* encodes the synthesis of indoleacetamide (IAM) from tryptophan, whereas *iaaH* encodes the conversion of IAM into IAA. The LHR strains of *A. vitis* lack the *ipt* genes normally found in *A. tumefaciens* WHR strains, and the introduction of this gene into an LHR strain leads to host range extension (13, 43). It was postulated that cytokinins inhibit tumor development on grapevine (110). The Ti plasmids of both WHR and LHR strains contain *iaa* genes. The *iaa* genes of Ag162 and Ag57 were found to be located on the TB-DNA (12, 110). Later it became clear that other *A. vitis* strains contain an active *ipt* gene and an additional active *iaaM* gene on their TA-region (6, 7) and are of the WHR type (76). Mutation studies of the TA- and TB-regions of the WHR plasmid pTiTm4 (45) showed that the *ipt* gene does indeed inhibit tumor formation when it is not counterbalanced by the combined action of the TB-*iaaM*, TA-*iaaH*, and TB-*iaaH* genes. Although the TB-*iaa* genes are sufficient to induce tumors on grapevine, their capacity to induce IAA synthesis is too weak to overcome cytokinin inhibition by the *ipt* gene (44). The TA-*iaaM* gene provides the necessary amount of IAM, which is converted to IAA by the TB-*iaaH* gene product. In the case of *ipt* gene loss (as has occurred in small TA O/C strains like AB3, Ag162, and Ag57), the TA-*iaaM* gene is no longer needed (and is also found to be deleted in these strains). These studies also revealed the oncogenic action of the TA-*6b* gene that induces tumor formation on grapevine in the absence of *ipt* and *iaa* genes (45). The mechanism of tumor formation by *6b* has not yet been determined, but active *6b* genes are present in all *A. vitis* strains. It was shown that the *6b* genes from different *Agrobacterium* strains have different tumor-inducing capacities and may therefore play a role in host range.

T-DNA Structures

Partial or complete T-DNA sequences have been determined for pTiTm4 (TA and TB), pTiAB3 (TA and TB), pTiAB4, pTiS4, and pTiCG474. Knowledge of these

TABLE 2 References for T-DNA maps of *A. vitis* model strains

Region of T-DNA	Type of Ti plasmid (strains)	Reference
Large TA	O/C (Tm4)	(74)
Large TA	O/C (Hm1)	(78)
Small TA	O/C (AB3, Ag57)	(74)
TB	O/C (Tm4, K305, Ag57, AB3, NW233, Hm1)	(37, 71)
Entire T region	N (AB4)	(65)
T1, T2, T3	V (S4)	(26)

sequences has greatly increased our understanding of T-DNA structures in *A. vitis* and facilitates the design of PCR primers for rapid detection and characterization of tumorigenic strains (87). Molecular studies of a large number of *A. vitis* strains has revealed four major types of T-DNA structures (summarized in Figure 1; references for maps in Table 2).

1. The large TA-DNAs of the O/C strains are clearly related to the classical octopine TL-DNA from *A. tumefaciens* strain A6. However, unlike the A6 TL-DNA, they have an intact and functional agrocinopine synthase (*acs*) gene (77), lack the *6a* gene, and carry an IS*866* element within the *iaaH* gene (except strain Hm1) (75). The small TA-regions (73–75) were derived from a large TA-region by an internal deletion that removed the *acs*, *iaaH*, *iaaM*, and *ipt* genes, leaving only the *6b* and *ocs* genes intact. Two additional IS elements, IS*868* and IS*869*, occupy more than one half of the small TA-DNA. The *A. vitis* octopine synthase enzymes also differ in biosynthetic capacities from the A6 enzyme in that they synthesize octopine, lysopine, and octopinic acid but not histopine and methiopine (70).

The Ti plasmids of the *A. vitis* O/C strains harbor a second T-region of about 20 kb, called the TB-region (12, 110). This region carries functional *iaa* genes, an *acs* gene, and the cucumopine synthase (*cus*) gene (also found in some *A. rhizogenes* strains). Cucumopine is a condensation product of histidine and α-ketoglutarate (31). The TB-DNA has not yet been fully sequenced, and it may contain other oncogenes or opine genes. At least six TB-DNA variants have been defined that have insertion elements at different positions (71). The TB-region of Tm4 is shown in Figure 1.

In general, the structures of the *A. vitis* TA and TB-regions have diverged considerably by acquisition of insertion elements like IS*866*, IS*867*, IS*868*, IS*869*, and IS*870* that are also found at other locations in the genome (both in the Ti plasmids and in the chromosomes). They may be associated with *A. vitis* or in a more general way with the Rhizobiaceae. Numbers and positions of these elements can be used to reconstruct the evolution of both plasmids and chromosomes (78). Evidence for clonal strain origin is provided by a detailed study of the TA-DNA

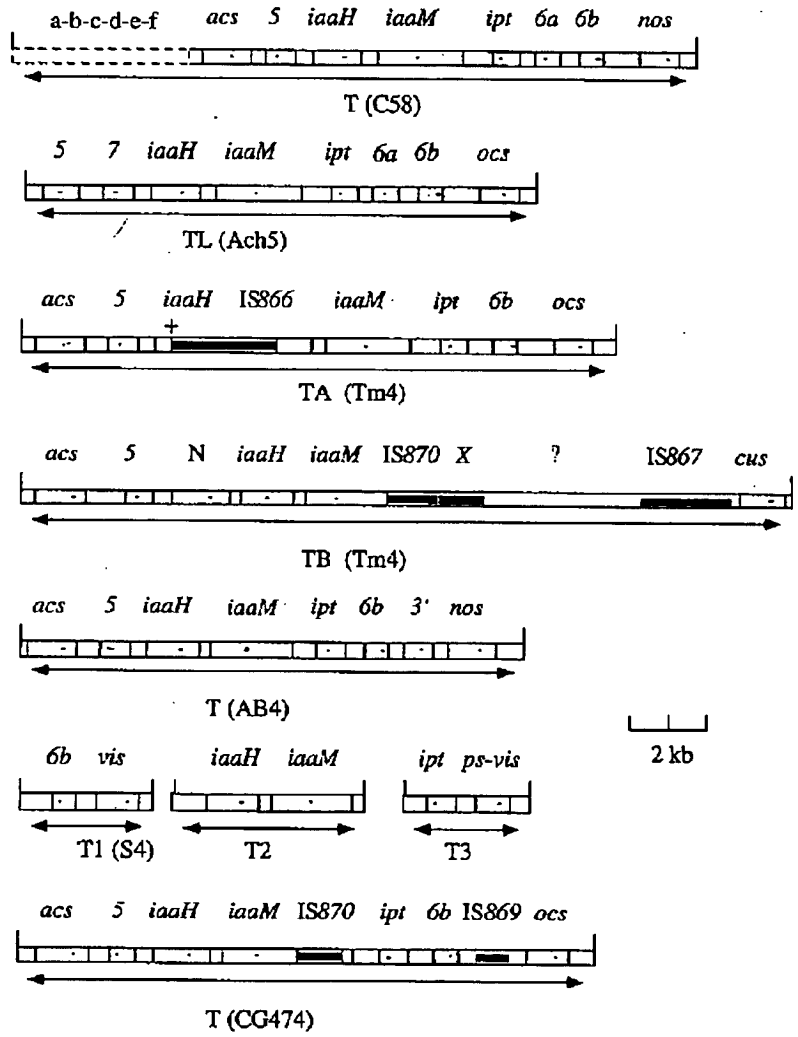

Figure 1 Structural differences of *A. tumefaciens* and *A. vitis* T-DNAs. From top to bottom: TA-DNA from nopaline (C58) and octopine (Ach5) *A. tumefaciens* strains. T-DNAs from *A. vitis* strains include TA-DNA from the octopine/cucumopine strain Tm4; TB-DNA from Tm4; T-DNA from the nopaline strain AB4; T1, T2, and T3 from vitopine strain S4; T-DNA from the octopine strain CG474. Sequenced regions are indicated by *double-headed arrows*.

of the O/C strain Hm1. This TA-DNA is very similar to the O/C large TA-DNAs but carries an intact, biologically active TA-*iaaH* gene (76). This suggests that the other WHR large TA O/C strains, all of which contain an IS*866* element inserted at the same position within the TA-*iaaH* gene, are derived from a single mutant that arose recently. Since O/C strains with large TA-regions have been isolated from vineyards in widely different parts of the world, it appears that this mutant has been effectively disseminated (possibly through trade of contaminated plant material). Note that the TA-*iaaH* gene interruption might be selectively neutral and linked to an unidentified selective character. Recent clonal origins have also been proposed for subtypes of the O/C Ti plasmids (37), the *A. vitis* nopaline (65), and vitopine (39) Ti plasmids.

2. The nopaline T-DNA of AB4 is found without variation in most *A. vitis* nopaline strains and is similar to the right-hand part of the classical nopaline T-DNA of *A. tumefaciens* C58. It lacks the 11.5-kb left-hand fragment of the pTiC58 T-DNA containing genes *a–f*. The functions of genes *a–f* are unknown, but gene *e* plays an important role in tumor induction by C58 (11). Apparently, AB4 can compensate for the loss of gene *e*.

Genes *6a* and *6b* found on the C58 T-DNA are replaced in AB4 by a fragment (F) carrying a distantly related *6b* gene and a gene called *3'* (the latter was first identified on the TR-DNA of *A. tumefaciens* octopine strains such as A6). Both genes have oncogenic properties (L Otten, unpublished data). Since a complete, C58-like nopaline T-DNA with fragment F has been found in pTi82139 (carried in *A. rhizogenes* strain 82139) (33), and since the *vir* region of AB4 to the left of the T-DNA is very similar to the O/C *vir* region, the *A. vitis* nopaline T-DNA probably results from a fusion between a pTi82139-like T-DNA and an O/C large TA-DNA. The precise fusion point has not yet been established.

3. The pTiS4 plasmid is unusual in that it contains three T-DNAs, the T1-, T2-, and T3-DNA, each with a single type of oncogenic function (*6b*, *iaaH/iaaM*, and *ipt*, respectively (26). The homology of these genes with their counterparts in other Ti plasmid types is surprisingly low, ranging from 58 to 62% of sequence identity. In contrast, homologies between genes from other T-DNAs is about 90–95%. This suggests that the T-DNA oncogenes have evolved over a longer period than was originally suspected. The T1-DNA carries the vitopine synthase (*vis*) gene, which is related to the octopine synthase gene. The structure of vitopine has not yet been determined.

4. Finally, the T-DNA of the unique isolate CG474 resembles the classical octopine TL-DNA and O/C TA-DNA (L Otten, unpublished data) but has a number of characteristic differences. It carries an IS*870* element in the *iaaM* gene (thus inactivating *iaa* synthesis) and an IS*869* element between the *6b* and *ocs* genes. The *acs* gene is intact and *6a* is missing. It is remarkable that this strain induces tumors on grapevine, in spite of an active *ipt* gene and the lack of an *iaa* system. Possibly, the *ipt* gene is less active than in the large TA O/C strains or is rendered less toxic by the *6b* gene. Several *6b* genes facilitate tumor formation in the presence of an inhibitory *ipt* gene (102).

Tartrate Utilization Plasmids

Most *A. vitis* strains degrade tartrate, an abundant compound in grapevine. Tartrate utilization can easily be tested by growth on minimal medium with 0.5% tartrate. It has been shown by conjugational plasmid transfer and DNA transformation that utilization of tartrate is plasmid-encoded in most of the strains (99). Three tartrate plasmid types, all of which are conjugative in planta, have been defined; pTrAB3 (245 kb), pTiAB3 (234 kb), and pTrAB4 (170 kb). pTrAB3 integrates readily into the chromosome of an acceptor strain together with another 60-kb plasmid from AB3 (64). The importance of this phenomenon under natural conditions is unknown but may lead to stabilization of the plasmid. The restriction maps of the tartrate utilization plasmids have been determined and the essential tartrate degradation regions (called TAR-I for pTrAB3, TAR-II for pTiAB3, and TAR-III for pTrAB4) have been analyzed by transposon mutagenesis using the *uidA*-Tn*5* transposon, which allows detection of transcriptional units. The TAR regions are similar in sequence (81); they are 11 kb in size and carry, among other genes, two tartrate dehydrogenase (*ttuC*) genes that were identified on the basis of homology to a *Pseudomonas ttuC* homologue. Like Ti plasmids, TAR regions are generally associated with characteristic chromosomal backgrounds (81). A few *Agrobacterium* grapevine strains use tartrate but lack classical TAR sequences, which indicates the existence of other tartrate utilization systems (81). Figure 2 presents the map and the functional organization of the TAR-I region of pTrAB3.

Remarkably, the three TAR regions found on otherwise unrelated plasmids are possibly the result of horizontal DNA transfer. The ends of the TAR regions show no evidence for insertion sequences so that the *ttu* genes do not seem to be part

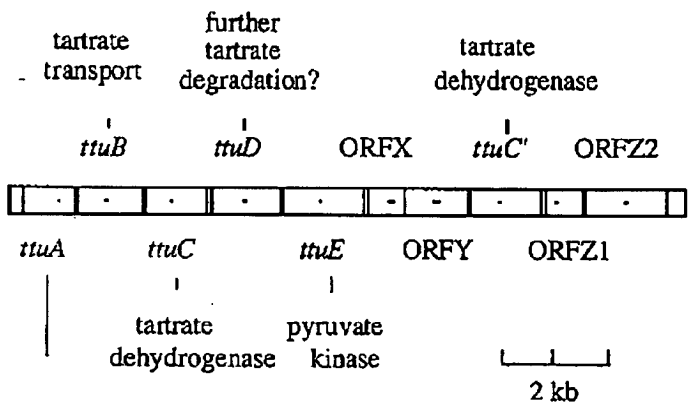

Figure 2 Structure and function of *A. vitis* tartrate utilization region, TAR-I (81, 82). This region is highly similar to that of TAR-II and TAR-III.

of a transposon. O/C strains with a small TA-DNA, like AB3, carry a pTrAB3-like plasmid with the TAR-I region and a Ti plasmid with a second TAR region (TAR-II). The fact that the pTiAB3-like plasmids carry a TAR region strongly suggests that exploitation of grapevine tissues through tartrate utilization and tumor formation is part of the same strategy and that such a Ti plasmid may constitute an efficient "grapevine colonization package." Indeed, a few *A. tumefaciens* strains isolated from grape carry a pTiAB3-like plasmid, the only type to contain both functions. Furthermore, competition experiments done in planta with one such strain and its tartrate-minus derivative showed that the TAR region provides a selective advantage for grape colonization (82).

Present evidence therefore suggests that tartrate utilization provides a competitive advantage to *A. vitis*. However, it was recently determined that most (23 of 26 strains) of nontumorigenic *A. vitis* isolated from feral grapevines do not utilize tartrate, which suggests that these strains possess other host colonization factors (22). It would be interesting to know whether other grape-associated bacteria, like *Xanthomonas ampelina*, carry tartrate plasmids and similar tartrate utilization genes.

Chromosomal Genes

Other potential host specificity–related factors include the production of polygalacturonase (PG) and endoglucanase (60). PG, which has been studied more extensively, is associated with induction of grape necrosis and was identified as a virulence factor since a PG-minus mutant was impaired in ability to induce tumors on grape and to attach to grape roots (10, 80). The *pehA* gene from *A. vitis*, which encodes for PG, was cloned and sequenced and the enzyme was compared to PGs from other microorganisms. The *A. vitis* enzyme is more similar to PGs produced by the plant pathogenic bacteria *Ralstonia solanacearum* and *Erwinia carotovora* than it is to those from *Aspergillus niger* and *Lycopersicum esculentum* (42) The *A. vitis* PG released dimers, trimers, and monomers from polygalacturonic acid and caused less electrolyte leakage from potato tubers than did PGs from *E. carotovora* and *R. solanacearum*.

Another chromosomal gene of *A. vitis* that may be associated with host interactions is homologous to *rfbC*, which encodes dTDP-rhamnose dehydratase that may be associated with EPS capsule biosynthesis (TC Herlache & TJ Burr, unpublished data). Primers derived from this gene amplify a 167-bp product from all *A. vitis* strains but not from *A. tumefaciens* or *A. rhizogenes* (61). Although its role in host interactions has not been identified, it is speculated that this gene may be associated with attachment of *A. vitis* to grape.

Although much progress has been made toward identifying genes that are associated with *A. vitis*-grape interactions, further research is needed to determine more specifically their roles under natural conditions. For example, the genetic makeup of LHR and WHR *A. vitis* T-DNAs is known and phenotypes can be differentiated on indicator plants. However, how do differences in T-DNAs affect grape host range or other interactions under natural conditions where pathogenicity of both groups appears to be limited to grape? Another example is gene *pehA*, which encodes a PG that is hypothesized to facilitate *A. vitis* attachment and systemic colonization of grape. However, nontumorigenic *A. tumefaciens* strains do not carry the *pehA* gene and are also common grape endophytes (TJ Burr, unpublished data).

Other observations also illustrate the difficulty of utilizing controlled inoculation experiments to study how Agrobacterium interacts with host plants in nature. For example, although tumorigenic *A. tumefaciens* are rarely isolated from grape crown galls, the bacterium causes large galls on grape shoot explants in vitro, even on cultivars known to be crown gall resistant (TJ Burr, unpublished data). In contrast, when grape shoot explants are inoculated with *A. vitis*, necrosis develops in 24–48 h (discussed below). Therefore, identifying genes and their functions that confer natural host specificity will be a great challenge but should provide key information to develop novel approaches for disease control.

A. VITIS-INDUCED GRAPE NECROSIS

A. vitis typically causes gall formation on grape trunks at or above graft unions. It is most interesting that galls are rarely observed on roots, but instead the bacterium induces a grape-specific necrosis. Tumorigenic and nontumorigenic *A. vitis* strains induce necrosis within 24–48 h on roots of all *Vitis* species, but not on other plants that have been examined (15, 95). Recently, it has been determined that necrosis is also induced on shoot explants and grape leaves, that induction is inoculum dependent, and that genes for necrosis are carried on the bacterial chromosome (TJ Burr, unpublished data). Therefore, *A. vitis*–induced necrosis is different from the necrosis reported for certain *A. tumefaciens* strains, which appears to be related to hormone toxicity and is associated with different Ti plasmid genes (79, 110).

As mentioned above, PG has been identified as one necrosis factor. A PG-minus derivative of strain CG49 (CG50) was shown to induce less necrosis at concentrations of 10^6 cfu/ml. Since higher concentrations of CG50 induced necrosis, it was concluded that factors other than PG were involved. Recently, it was shown that a *pehA*-complemented CG50 produced more necrosis than the noncomplemented mutant, but significantly less than CG49 (TC Herlache & TJ Burr, unpublished data). These results have encouraged further research on the necrosis mechanism.

It was recently discovered that *A. vitis* induces a hypersensitive response (HR) on tobacco and that both HR and grape necrosis can be blocked by preinoculation with certain plant metabolic inhibitors and may be mechanistically related (TC Herlache & TJ Burr, unpublished data). Necrosis and HR reactions therefore require active plant metabolism. Like HR reactions of other gram-negative bacteria, the *A. vitis* HR has an induction period in tobacco (8–12 h) prior to which the reaction can be stopped by infiltrating leaf panels with antibiotics. HR- and necrosis-associated genes are being cloned and sequenced.

SURVIVAL IN SOIL AND PLANT TISSUE

Although bacteria in the genus *Agrobacterium* are common soil inhabitants, tumorigenic strains are almost exclusively detected in association with galls and plant residues in soil (3, 17). Following infestation of soil with *A. vitis*–colonized grape roots and canes, the bacterium was detected in the tissues for at least two years (25). The roots and canes did not have galls, however, although all of the recovered strains were still tumorigenic and carried O/C, N, or V-type Ti plasmids. Therefore the bacteria are able to maintain their Ti plasmids while persisting in an apparent saprophytic state in decaying grape tissues. In another study where grapes or oats were planted in soils infested with *A. vitis*, the bacterium maintained greater populations in grape as compared with oat rhizospheres (5). These and other studies infer that *A. vitis* may not persist in soils in the absence of grape tissues and that the bacterium is primarily introduced into soils with contaminated grape plants. However, because the sensitivities of methods for detecting the bacterium in soil are low (generally populations $< 10^2$–10^3 cfu/g soil cannot be detected) and because of the recent findings that are discussed below, further investigations of soil survival are necessary.

As mentioned previously, studies were undertaken in the United States (22) and Italy (C Bazzi, unpublished data) to determine if *A. vitis* persists in feral *Vitis* spp. *A. vitis* was detected from 41 of 66 root samples collected from regions near to and far removed from commercial vineyards in New York and Vermont. All strains that were identified as *A. vitis* were nontumorigenic and were genetically diverse, as determined by DNA fingerprinting. In Italy, over 50 strains of nontumorigenic *A. vitis* were recovered from cuttings taken from feral *V. vinifera silvestris* vines. Since the feral vines probably grew from seeds that were disseminated by animals, one must question the source of *A. vitis* on them. It may be that *A. vitis* is a relatively common inhabitant of soils, but can only be detected by conventional plating methods at populations achieved in association with grapevines. Further research is therefore needed to determine if *A. vitis* survives in soil at low population densities. The ability to make this determination will be, at least partially, dependent on the development of detection methods having increased sensitivities.

Important questions remain on the role of feral grapevines in the survival of *A. vitis* and the spread of crown gall disease. Do tumorigenic strains colonize feral grapevines, and can the nontumorigenic strains that are present acquire and maintain Ti plasmids? To answer the second question, we mated tumorigenic strains (carrying O/C and N Ti plasmids) with two nontumorigenic strains from feral *V. riparia* and with one from *V. vinifera* (TJ Burr, unpublished data). Matings were done on grape galls that were caused by the donor strain (to assure the presence of proper opines). Equal concentrations of donor and recipient (rifampicin resistant) were incubated on galls, and selection for Ti plasmid transfer was done on media containing rifampicin and the appropriate opine as the sole N and C source. From six matings (338 colonies selected from opine media), no stable Ti plasmid transfer to strains from *V. riparia* was detected, and the O/C Ti plasmid was transferred to a strain of *V. vinifera* in one mating. In two cases, evidence of a transferred Ti plasmid was initially detected by Southern analysis, however after further evaluation the plasmid could no longer be detected. Additional research is needed to identify factors that are associated with the apparent inhibition of Ti plasmid transfer. Interestingly, transconjugants were frequently identified that had gained a plasmid (other than Ti) that conferred tartrate utilization from donor strains.

Endophytic Survival

Lehoczky first demonstrated that *A. vitis* survives endophytically in apparently healthy grapevines and initiates gall formation at injury sites (57). In a series of papers, he demonstrated the presence of the bacterium in grape sap (58) and hypothesized that bacterial cells concentrate in the grape root system in winter and move into the upper parts of the vine during sap flow (59). In fact, *A. vitis* is routinely isolated from root tissues, even during the growing season; however, the frequency of isolation from roots has not been compared with that from other tissues. Although it has been generally assumed that *A. vitis* persists primarily in xylem tissues, at least two studies indicate that the bacterium may reside in tissues other than the xylem. Süle reported that the highest *A. vitis* populations were detectable in phloem tissue (93), and Jager et al (47) provided evidence that the bacterium persists in the rind layer of dormant cuttings (directly below the bark).

Bauer et al examined the seasonal distribution of *A. vitis* in vines in Germany (1). In this case, *A. vitis* was detected primarily at the inoculation sites on canes and did not readily disseminate throughout the vine. By using antibiotic-marked strains it was, however, possible to detect *A. vitis* cells that migrated from the inoculation points to roots within 15 weeks. Populations at inoculation sites showed seasonal fluctuations (they were highest in late May and in October) and differed between cultivars; they were highest in Riesling as compared with Müller-Thurgau. Stover et al also found that *A. vitis* does not readily migrate from inoculation points and that populations differed between resistant and susceptible cultivars (91). He also showed that freezing of canes facilitates the systemic movement of *A. vitis* (a 41,000-fold increase in cells was detected after flushing water through freeze-treated canes). Since there were no differences in total numbers of cells detected in frozen vs nonfrozen canes, it was concluded that freezing played a physical role in allowing the bacteria to move within the cuttings. If one considers that *A. vitis* may reside in phloem or rind tissues, then freeze injuries may facilitate its spread into xylem tissues and enhance dissemination within the vine. The finding that freezing facilitates systemic movement has potential implications for improving the sensitivity of indexing methods (discussed below). It also suggests that freezing injuries may be important not only for stimulating the secretion of *Agrobacterium* virulence gene inducers from injured cells (discussed below), but may also facilitate the internal movement of the bacterium in vines.

MANAGEMENT OF GRAPE CROWN GALL

Grape growers have dealt with crown gall disease for over 100 years and recognized early on that cultivars differ in susceptibility to disease and to factors that cause vine injury, particularly freezing temperatures, which stimulates disease development (41). Some cultural practices that can affect vine injury and thus crown gall development include vineyard site selection, crop and canopy management, and the protection of trunks with soil during winter months. When practical, the selection of resistant rootstock and scion cultivars can also greatly influence disease development. Vitis germplasms have been evaluated for crown gall susceptibility by different research groups (36, 90); in general, rootstocks of *V. riparia* and *V. rupestris* parentage are more resistant than *V. vinifera* cultivars. The genetic basis for resistance was investigated by screening progeny from crosses between resistant (*V. amurensis*) and susceptible cultivars. Following inoculation with *A. vitis* strain AT-1, it was concluded that resistance is controlled by a single dominant gene (97). It remains to be determined if the same pattern of resistance will be observed on other grape progeny when inoculated with a genetically diverse collection of *A. vitis* strains.

Süle et al (94) compared crown gall severity under field conditions on a highly susceptible scion cultivar, Blue Frank, that was grafted onto either crown gall–resistant (Riparia 'Gloire') or –susceptible (Teleki 5C) rootstocks. Over a six-year period, significantly more crown gall and vine death occurred on vines that were grafted on the susceptible rootstock. This report is particularly significant in that it is the first under controlled conditions in the field to demonstrate a beneficial effect of using crown gall–resistant rootstocks. The reduction in disease may be related to reduced survival of *A. vitis* in resistant as compared with susceptible grape genotypes (91). Other research on crown gall management has focused on developing methods to index vines, producing propagation material that is free of the pathogen, and on biological control. A review of disease management strategies was recently published (14).

Indexing for *A. vitis*

Lehoczky demonstrated the presence of *A. vitis* in symptomless dormant cuttings by isolating the bacterium from callus tissue that formed at the base of cuttings (58). Variations of this method have been used successfully by others to index cuttings (93), although its sensitivity has not been determined. Another indexing method, developed by Tarbah & Goodman (100) and by Bazzi et al (2), involves forcing water or buffer through cuttings with vacuum pressure and isolating the pathogen from the collected extracts. *A. vitis* is then identified by plating the extracts on semiselective media (18) and/or by serology (4). The efficiency of the method was evaluated by infiltrating cuttings with known concentrations of *A. vitis* and then calculating the percentage of recovered cells (2). Because only about 12% of the introduced cells were detected, the method would appear to have limited value for general indexing of propagation wood sources. As mentioned above, freezing of cuttings prior to flushing with water greatly increases the recovery of *A. vitis* and therefore may significantly improve the efficiency of this method.

Increased emphasis has been placed on the use of DNA probes and PCR (32) to identify *Agrobacterium* spp. Probes have consisted of chromosomal and T-DNA genes and IS elements (20, 63, 87). Sawada et al (83) compared primers derived from conserved regions of *virG*, *virC1*, and *virC2*. A primer pair including a forward primer (VCF) from *virC1* and a reverse primer (VCR) from *virC2* consistently gave a 730-bp PCR product from 75 of 77 strains of tumorigenic species including *A. tumefaciens*, *A. rhizogenes*, *A. rubi*, and *A. vitis*. When other primers consisting of forward primers from *virG* and reverse from *virC* genes were used, some strains did not yield products and others yielded products of unexpected sizes.

Similarly, Haas et al developed primers derived from the endonuclease portion of the *virD2* gene and from a conserved region of the *ipt* gene (40). In this case, 44 strains representing *A. tumefaciens*, *A. rhizogenes*, and *A. vitis* all yielded the expected 338-bp product from the *virD2* primer, whereas nontumorigenic strains did not. The *ipt* primer pair also produced an expected product size from tumorigenic strains but, as expected, not from rhizogenic strains or from a LHR *A. vitis* strain. Using pure cultures of bacterial cells, this PCR procedure could detect reaction mixtures containing as few as 150–200 cells.

There is continued development of PCR-based methods for *A. vitis* and other *Agrobacterium* spp. that utilize specific primers and novel methods for isolating bacteria and bacterial DNA from plants and soil. Eastwell et al (35) tested different methods of extracting bacterial DNA from dormant cuttings and compared primers for their effectiveness in detecting *A. vitis*. Primers were derived from the *pehA* gene (polygalacturonase gene) and from *virA* of the Ti plasmid. The method was effective in detecting *A. vitis* in grape cuttings collected from grapevines expressing crown gall. In this case, *pehA* primers amplify a characteristic product from all *A. vitis* (tumorigenic and nontumorigenic), and *virA* primers amplify a characteristic product from *A. vitis* strains carrying N and O/C but not V Ti plasmids. To overcome this problem, we recently developed a *virE2* primer that amplifies a characteristic product from strains with V Ti plasmids (61). The ability to detect vitopine strains in cuttings is essential because they make up a significant portion of *A. vitis* strains found in grape (68). The *pehA* primers are highly reliable for identifying *A. vitis*, although recently a naturally occurring PG-negative strain of *A. vitis* was isolated from *V. riparia* (TJ Burr, unpublished data). However, where a grape grower wishes to know if propagation material is contaminated with tumorigenic *A. vitis*, exclusive reliance on *pehA* primers will not be sufficient. Nontumorigenic strains, which carry *pehA*, are often encountered in *Vitis* spp., and at least some nontumorigenic strains may actually benefit the plant by preventing tumorigenic strains from causing infections (discussed below). In place of *pehA* primers, we recently evaluated primers derived from the *rfbC* homologue that was previously discussed (61).

Another recently developed method to detect *A. vitis* in grapevines involves the use of an immunocapture technique, followed by PCR (51). In this case, extracts from grape canes are incubated in antibody-coated tubes to which 3DG medium (selective for *A. vitis*) is subsequently added. DNA from the resulting bacteria is analyzed by PCR with primers derived from the *6b* gene of the *A. vitis* O/C strain Tm4. By this method, a characteristic PCR product was observed for samples known to be infected with *A. vitis* carrying O/C-type Ti plasmids. The authors suggested improvements by employing antibodies and PCR primers that would detect diverse types of *A. vitis*.

The major components of a method for efficient and sensitive detection of tumorigenic *A. vitis* in dormant cuttings seem to be in place. Further studies are needed to compare some of the most promising techniques described above and to compare PCR primers to assure that they will amplify characteristic products from the diverse genetic groups of *A. vitis*. It may be that *A. vitis*-specific primers, such as those from *pehA*, or derived from the *rfbC* gene together with universal primers for tumorigenicity, (such as those reported by Haas et al or Sawada et al) would be highly effective.

A. vitis–Free Propagation Material

The submersion of dormant grape cuttings in a 50°C water bath for 30 min to eradicate *A. vitis* was first reported in 1989 (21). Although initial findings indicated that treatments were effective, a more critical set of experiments demonstrated that low levels of the bacterium survived in tissues near galls even after treatments of 55°C for 30 min (23).

A significant drawback to the use of hot water treatments is the potential for bud injury. Factors including temperature and duration of treatment, the month when cuttings were collected (differences in state of dormancy), and pre- and posttreatment storage were evaluated on cultivar Cabernet Sauvignon by Wample in Washington State (105, 106). He demonstrated that cuttings collected in January and stored posttreatment had the greatest bud survival following hot water treatments. Good bud survival was achieved even following a treatment of 56°C. The use of such temperatures, which are greater than previously tested, to eradicate *A. vitis* may improve the efficacy, although this has not been evaluated. In contrast, using temperatures above 50°C on cuttings collected from vineyards in other areas, such as New York State, has led to significant bud kill (TJ Burr, unpublished data). The reasons for these apparent differences in bud sensitivity to treatments conducted in different geographic regions are unknown but may be related to cultivar differences or to wood hardiness. Therefore, the usefulness of hot water treatments as a crown gall management strategy needs further evaluation. Recent findings suggest that hot water treatments may be warranted even if they do not completely eliminate *A. vitis*. For example, it was observed in Italy and in a commercial nursery in Washington State that hot water–treated cuttings have enhanced callus and root formation, which promotes better plant growth (C Bazzi & TJ Burr, unpublished data). Such benefits have resulted in the routine treatment of several million cuttings annually in some nurseries.

The only proven way of producing *A. vitis*-free grapevines is by propagating vines from shoot tips in vitro. *A. vitis* does not systemically invade green grape shoots (19, 98), and therefore it is possible to exclude the bacterium by shoot tip culture. This practice has been used by at least one commercial vineyard to establish a crown gall–free mother block that has remained free of crown gall for over five years. Establishment of such a planting requires the selection of a site where the soil is not contaminated with *A. vitis*. Problems of assuring that soils are free of the bacterium were discussed previously.

Biological Control

Several laboratories have attempted to identify biological control agents for grape crown gall (54; reviewed in 14). One promising strain, F2/5, is a nontumorigenic *A. vitis* that was isolated in South Africa (89). F2/5 produces an antibiotic that inhibits growth of many tumorigenic *A. vitis* strains in vitro; however, antibiotic-minus mutants of F2/5 were found to be as effective as the wild-type strain for controlling crown gall (24). We are continuing to study the mechanism(s) by which F2/5 inhibits tumorigenesis on grape. Some nontumorigenic *A. vitis* strains from feral *V. riparia* were as effective as F2/5 for inhibiting crown gall caused by strain K306 (22). Subsequently, one strain, CG523, was compared with F2/5 for controlling several tumorigenic strains representing the major *A. vitis* groups. Biological control agents were applied to inoculation sites either 24 h before or simultaneously with the pathogen. F2/5 was more effective for inhibiting gall formation than was CG523. Although some of the tumorigenic strains were not inhibited by F2/5 following simultaneous inoculations, control was enhanced if F2/5 was applied 24 h prior to the pathogen.

Genetic Engineering for Crown Gall Resistance

In recent years, the ability to genetically transform *Vitis* spp. has greatly improved (56), and the prospects for using genetic engineering to confer resistance to bacterial pathogens in plants look promising (34). Because crown gall is an important disease with few disease control options, the use of transgenic approaches for control is an attractive alternative. A main goal will be to engineer resistance into long-standing, high-quality wine cultivars that are crown gall susceptible and are not likely to be replaced by conventional breeding. One strategy currently being tested involves expressing a deletion mutant of the Ti plasmid *virE2* gene in plants. It was previously determined that expression of some *virE2* mutant genes in tobacco results in plants that are resistant to infection by *A. tumefaciens*, possibly as a result of inhibition of T-strand nuclear import (30). Following the expression of a *virE2* mutant in grape, transgenic lines with significant resistance to crown gall were identified (TJ Burr, unpublished data). Other transgenic strategies for crown gall resistance are sure to follow.

CONCLUSIONS

Although crown gall continues to be a serious problem on grape, significant progress has been made toward understanding pathogen biology and disease control. Characterization of worldwide collections of *A. vitis* has provided significant insight into genetic diversity within the species and its relatedness to other Rhizobiacea. Substantial Ti plasmid sequence information has further enhanced our understanding of diversity within the bacterium and has allowed the prediction of phylogenetic relationships. Sequencing has also facilitated the identification of specific genes that are essential for tumorigenicity and other bacterium-host interactions. Sequence information, together with PCR-RFLP protocols, make it possible to develop specific detection methods, which, in addition to having commercial value, will be essential to answer important epidemiological questions related to pathogen survival and spread in nature. For example, does *A. vitis* survive in soil? How do nontumorigenic strains differ from tumorigenic strains? Can nontumorigenic strains from feral grapevines acquire Ti plasmids? If not, what factors affect plasmid transfer?

In comparison with the genetic makeup of Ti plasmids, much less is known about specific chromosomal genes and their functions. DNA fingerprint comparisons have revealed that *A. vitis* chromosomes are diverse and are highly correlated with the Ti plasmid type carried in strains. Further research is needed to determine the roles of chromosomal genes that are associated with phenotypes such as grape necrosis, tobacco HR, and the ability of some nontumorigenic strains to inhibit crown gall infections.

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LITERATURE CITED

1. Bauer C, Schulz TF, Lorenz D, Eichhorn KW, Plapp R. 1994. Population dynamics of *Agrobacterium vitis* in two grapevine varieties during the vegetation period. *Vitis* 33:25–29
2. Bazzi C, Piazza C, Burr TJ. 1987. Detection of *Agrobacterium tumefaciens* in grapevine cuttings. *Bull. OEPP* 17:105–12
3. Bien E, Lorenz D, Eichhorn K, Plapp R. 1990. Isolation and characterization of *Agrobacterium tumefaciens* from the German vine region Rheinpfalz. *Z. Pflanzenkr. Pflanzenschutz* 97:313–22
4. Bishop AL, Burr TJ, Mittak VL, Katz BH. 1989. A monoclonal antibody specific to *Agrobacterium tumefaciens* biovar 3 and its utilization for indexing grapevine propagation material. *Phytopathology* 79:995–98
5. Bishop AL, Katz BH, Burr TJ. 1988. Infection of grapevines by soilborne *Agrobacterium tumefaciens* biovar 3 and population dynamics in host and nonhost rhizospheres. *Phytopathology* 78(7):945–48
6. Bonnard G, Tinland B, Paulus F, Szegedi E, Otten L. 1989. Nucleotide sequence, evolutionary origin and biological role of a rearranged cytokinin gene isolated from a wide host range biotype III Agrobacterium strain. *Mol. Gen. Genet.* 216:428–38
7. Bonnard G, Vincent F, Otten L. 1991. Sequence of *Agrobacterium tumefaciens* biotype III auxin genes. *Plant Mol. Biol.* 16:733–38
8. Bouzar H, Jones JB, Hodge NC. 1993. Differential characterization of Agrobacterium species using carbon-source utilization patterns and fatty acid profiles. *Phytopathology* 83:733–39
9. Braun AC, Mandle RJ. 1948. Studies on the inactivation of the tumor inducing principle in crown gall. *Growth* 12:255–69
10. Brisset MN, Rodriguez Palenzuela P, Burr TJ, Collmer A. 1991. Attachment chemotaxis and multiplication of *Agrobacterium tumefaciens* biovar 1 and biovar 3 on grapevine and pea. *Appl. Environ. Microbiol.* 57:3178–82
11. Broer I, Droge-Laser W, Barker RF, Neumann K, Klipp W. 1995. Identification of the *Agrobacterium tumefaciens* C58 T-DNA genes e and f and their impact on crown gall tumour formation. *Plant Mol. Biol.* 27:41–57
12. Buckholtz WB, Thomashow MF. 1984. Comparison of T-DNA oncogene complements of *Agrobacterium tumefaciens* tumor-inducing plasmids with limited and wide host ranges. *J. Bacteriol.* 160:319–26
13. Buckholtz WB, Thomashow MF. 1984. Host range encoded by the *Agrobacterium tumefaciens* tumor-inducing plasmid can be expanded by modification of its T-DNA oncogene complement. *J. Bacteriol.* 160:327–32
14. Burr TJ, Bazzi C, Süle S, Otten L. 1998. Crown gall of grape: biology of *Agrobacterium vitis* and the development of disease control strategies. *Plant Dis.* 82:1288–97
15. Burr TJ, Bishop AL, Katz BH, Blanchard LM, Bazzi C. 1987. A root-specific decay of grapevine caused by *Agrobacterium tumefaciens* and *A. radiobacter* biovar 3. *Phytopathology* 77:1424–27
16. Burr TJ, Bishop AL, Katz BH, Blanchard LM, Bazzi C. 1987. A root-specific decay of grapevine caused by *Agrobacterium tumefaciens* and *Agrobacterium radiobacter* biovar 3. *Phytopathology* 77(10):1424–27
17. Burr TJ, Katz BH. 1983. Isolation of *Agrobacterium tumefaciens* biovar 3 from grapevine galls and sap and from vineyard soil. *Phytopathology* 73:163–65
18. Burr TJ, Katz BH, Bishop AL. 1987. Populations of Agrobacterium in vineyard and nonvineyard soils and grape roots in vineyards and nurseries. *Plant Dis.* 71:617–20
19. Burr TJ, Katz BH, Bishop AL, Meyers CA, Mittak VL. 1988. Effect of shoot age and tip culture propagation of grapes on systemic infestations by *Agrobacterium tumefaciens* biovar 3. *Am. J. Enol. Vitic.* 39:67–70
20. Burr TJ, Norelli JL, Katz BH, Bishop AL. 1990. Use of Ti-plasmid DNA probes for determining tumorigenicity of Agrobacterium strains. *Appl. Environ. Microbiol.* 56:1782–85
21. Burr TJ, Ophel K, Katz BH, Kerr A. 1989. Effect of hot water treatment on systemic *Agrobacterium tumefaciens* biovar 3 in dormant grape cuttings. *Plant Dis.* 73:242–45
22. Burr TJ, Reid CL, Adams CE, Momol EA. 1999. Characterization of *Agrobacterium vitis* strains isolated from feral *Vitis riparia*. *Plant Dis.* 83:102–7
23. Burr TJ, Reid CL, Splittstoesser DF, Yoshimura M. 1996. Effect of heat treatments on grape bud mortality and survival of *Agrobacterium vitis* in vitro and in dormant grape cuttings. *Am. J. Enol. Vitic.* 47:119–23
24. Burr TJ, Reid CL, Tagliati E, Bazzi C, Süle S. 1997. Biological control of grape crown gall by strain F2/5 is not associated with agrocin production or competition for attachment sites on grape cells. *Phytopathology* 87(7):705–11
25. Burr TJ, Reid CL, Yoshimura M, Momol EA, Bazzi C. 1995. Survival and tumorigenicity of *Agrobacterium vitis* in living and decaying grape roots and canes in soil. *Plant Dis.* 79:677–82
26. Canaday J, Gerard JC, Crouzet P, Otten L. 1992. Organization and functional analysis

of three T-DNAs from the vitopine Ti plasmid pTiS4. *Mol. Gen. Genet.* 235:292–303
27. Cangelosi GAR, Ankenbauer G, Nester EW. 1990. Sugars induce the Agrobacterium virulence genes through a periplasmic protein and a transmembrane signal protein. *Proc. Natl. Acad. Sci. USA* 87:6708–12
28. Cavara F. 1895. Apercu sommaire de quelques maladies de la vigne parues en Italie en 1894. *Rev. Int. Vitic. Oenol.* 6:447–49
29. Chilton M-D, Drummond MH, Merlo DJ, Sciaky D, Montoya AL, et al 1977. Stable incorporation of plasmid DNA into higher plant cells: the molecular basis of crown gall tumorigenesis. *Cell* 11:263–71
30. Citovsky V, Warnick D, Zambryski P. 1994. Nuclear import of Agrobacterium VirD2 and VirE2 proteins in maize and tobacco. *Proc. Natl. Acad. Sci. USA* 91:3210–14
31. Davioud E, Petit A, Tate ME, Ryder MH, Tempe J. 1988. Cucumopine, a new T-DNA-encoded opine in hairy root and crown gall. *Phytochemistry* 27:2429–34
32. Dong LC, Sun CW, Thies KL, Luthe DS, Graves CHJ. 1992. Use of polymerase chain reaction to detect pathogenic strains of Agrobacterium. *Phytopathology* 82:434–39
33. Drevet C, Brasileiro ACM, Jouanin L. 1994. Oncogene arrangement in a shooty strain of *Agrobacterium tumefaciens*. *Plant Mol. Biol.* 25:83–90
34. Düring K. 1996. Genetic engineering for resistance to bacteria in transgenic plants by introduction of foreign genes. *Mol. Breed.* 2:297–305
35. Eastwell KC, Willis LG, Cavileer TD. 1995. A rapid and sensitive method to detect *Agrobacterium vitis* in grapevine cuttings using the polymerase chain reaction. *Plant Dis.* 79:822–27
36. Ferreira JHS, van Zyl FGH. 1986. Susceptibility of grape-vine rootstocks to strains of *Agrobacterium tumefaciens* biovar 3. *S. Afr. J. Enol. Vitic.* 7:101–4
37. Fournier P, De Ruffray P, Otten L. 1994. Natural instability of *Agrobacterium vitis* Ti plasmid due to unusual duplication of a 23-kb DNA fragment. *Mol. Plant-Microbe Interact.* 7:164–72
38. Deleted in proof
39. Gerard JC, Canaday J, Szegedi E, De La Salle H, Otten L. 1992. Physical map of the vitopine Ti plasmid $pTiS_4$. *Plasmid* 28:146–56
40. Haas JH, Moore LW, Ream W, Manulis S. 1995. Universal PCR primers for detection of phytopathogenic Agrobacterium strains. *Appl. Environ. Microbiol.* 61:2879–84
41. Hedgecock GG. 1910. Field studies of the crown-gall of the grape. *US Dep. Agric. Bur. Plant Ind. Bull.* 40 pp.
42. Herlache TC, Hotchkiss AT Jr, Burr TJ, Collmer A. 1997. Characterization of the *Agrobacterium vitis pehA* gene and comparison of the encoded polygalacturonase with the homologous enzymes from *Erwinia carotovora* and *Ralstonia solanacearum*. *Appl. Environ. Microbiol.* 63:338–46
43. Hoekema A, de Pater BS, Fellinger AJ, Hooykaas PJJ, Schilperoort RA. 1984. The limited host range of an *Agrobacterium tumefaciens* strain extended by a cytokinin gene from a wide host range T-region. *EMBO J.* 30:43–47
44. Huss B, Bomard G, Otten L. 1989. Isolation and functional analysis of a set of auxin genes with low root-inducing activity from an *Agrobacterium tumefaciens* biotype III strain. *Plant Mol. Biol.* 12:271–83
45. Huss B, Tinland B, Paulus F, Walter B, Otten L. 1990. Functional analysis of a complex oncogene arrangement in biotype III *Agrobacterium tumefaciens* strains. *Plant Mol. Biol.* 14:173–86
46. Irelan NA, Meredith CP. 1996. Genetic analysis of *Agrobacterium tumefaciens* and *A. vitis* using randomly amplified polymorphic DNA. *Am. J. Enol. Vitic.* 47(2):145–51
47. Jager J, Lorenz D, Plapp R, Eichhorn KW. 1989. Latent occurence of *Agrobacterium tumefaciens* biovar 3 in grapevine (*Vitis vinifera* L.). *Vitic. Enol. Sci.* 44:14–20
48. Jarvis BDW, Sivakumaran S, Tighe SW, Gillis M. 1996. Identification of Agrobacterium and Rhizobium species based on cellular fatty acid composition. *Plant Soil* 184:143–58
49. Jin S, Roitsch T, Akenbauer RG, Gordon MP, Nester EW. 1990. The Vir A protein of *Agrobacterium tumefaciens* is autophosphorylated and is essential for *vir* gene regulation. *J. Bacteriol.* 172:525–30
50. Kado CI. 1994. Promiscuous DNA transfer system of *Agrobacterium tumefaciens*: role of the virB operon in sex pilus assembly and synthesis. *Mol. Microbiol.* 12:17–22
51. Kaufmann M, Kassemeyer HH, Otten L. 1996. Isolation of *Agrobacterium vitis* from grapevine propagating material by means of PCR after immunocapture cultivation. *Vitis* 35(3):151–53
52. Kerr A. 1969. Transfer of virulence between isolates of Agrobacterium. *Nature* 233:1175–76
53. Kerr A, Panagopoulos CG. 1977. Biotypes of *Agrobacterium radiobacter* var. *tumefaciens* and their biological control. *Phytopathol. Z.* 90:172–79
54. Khmel IA, Sorokina TA, Lemanova NB, Lipasova VA, Metlitski OZ, et al 1998. Biological control of crown gall in grapevine and raspberry by two *Pseudomonas* spp. with a wide spectrum of antagonistic activity. *Biocontrol Sci. Technol.* 8:45–57
55. Knauf VC, Yanofsky M, Montoya A, Nester EW. 1984. Physical and functional map of an *Agrobacterium tumefaciens* plasmid that confers a narrow host range. *J. Bacteriol.* 160:564–68
56. Krastanova S, Perrin M, Barbier P, Demangea G, Cornuet P, et al 1995. Transformation of grapevine rootstocks with the coat protein gene of grapevine fanleaf nepovirus. *Plant Cell Rep.* 14:550–54
57. Lehoczky J. 1968. Spread of *Agrobacterium tumefaciens* in the vessels of the grapevine after natural infection. *Phytopathol. Z.* 63:239–46
58. Lehoczky J. 1971. Further evidences concerning the systemic spreading of *Agrobacterium tumefaciens* in the vascular system of grapevines. *Vitis* 10:215–21
59. Lehoczky J. 1978. *Root-system of the grapevine as a reservoir of Agrobacterium tumefaciens cells. Proc. 4th Int. Conf. Plant Pathol. Bacteriol.* Angers, pp. 239–43
60. McGuire RG, Rodriguez Palenzuela P, Collmer A, Burr TJ. 1991. Polygalacturonase production by *Agrobacterium tumefaciens* biovar 3. *Appl. Environ. Microbiol.* 57:660–64
61. Momol EA, Herlache TC, Burr TJ. 1998. Detection of *Agrobacterium vitis* in grape by PCR. *Phytopathology* 88(Suppl.) S64
62. Ophel K, Kerr A. 1990. *Agrobacterium vitis*, new species for strains of Agrobacterium biovar 3 from grapevines. *Int. J. Syst. Bacteriol.* 40:236–41
63. Otten L, Canaday J. 1992. Identification of closely related *Agrobacterium vitis* isolates by chromosomal DNA probes. *Microb. Releases* 1:29–34
64. Otten L, Crouzet P, Salomone JY, De Ruffray P, Szegedi E. 1995. *Agrobacterium vitis* strain AB3 harbors two independent tartrate utilization systems, one of which is encoded by the Ti plasmid. *Mol. Plant-Microbe Interact.* 8:138–46
65. Otten L, De Ruffray P. 1994. *Agrobacterium vitis* nopaline Ti plasmid pTiAB4: relationship to other Ti plasmids and T-DNA structure. *Mol. Gen. Genet.* 245: 493–505
66. Otten L, De Ruffray P. 1996. Major differences between the rrnA operons of two strains of *Agrobacterium vitis*. *Arch. Microbiol.* 166:68–70
67. Otten L, De Ruffray P, De Lajudie P, Michot B. 1996. Sequence and characterisation of a ribosomal RNA operon from *Agrobacterium vitis*. *Mol. Gen. Genet.* 251:99–107

68. Otten L, De Ruffray P, Momol EA, Momol MT, Burr TJ. 1996. Phylogenetic relationships between *Agrobacterium vitis* isolates and their Ti plasmids. *Mol. Plant-Microbe Interact.* 9:782–86
69. Otten L, Gerard JC, De Ruffray P. 1993. The Ti plasmid from the wide host range *Agrobacterium vitis* strain Tm4 map and homology with other Ti plasmids. *Plasmid* 29:154–59
70. Otten L, Szegedi E. 1985. Crown galls induced by octopine-degrading biotype 3 strains of *Agrobacterium tumefaciens* contain a new form of lysopine dehydrogenase. *Plant Sci.* 40:81–85
71. Otten L, van Nuenen M. 1993. Natural instability of octopine/cucumopine Ti plasmids of clonal origin. *Microb. Releases* 2:91–96
72. Panagopoulos CG, Psallidas PG. 1973. Characteristics of Greek isolates of *Agrobacterium tumefaciens* (E.F. Smith & Townsend) Conn. *J. Appl. Bacteriol.* 36: 233–40
73. Paulus F, et al 1991. Role of T-region borders in Agrobacterium host range. *Mol. Plant-Microbe Interact.* 4:163–72
74. Paulus F, Canaday J, Otten L. 1991. Limited host range Ti plasmids: recent origin from wide host range Ti plasmids and involvement of a novel IS element, IS868. *Mol. Plant-Microbe Interact.* 4:190–97
75. Paulus F, Canaday J, Vincent F, Bonnard G, Kares C, Otten L. 1991. Sequence of the iaa and ipt region of different *Agrobacterium tumefaciens* biotype III octopine strains: reconstruction of octopine Ti plasmid evolution. *Plant Mol. Biol.* 16:601–14
76. Paulus F, Huss B, Bonnard G, Ride M, Szegedi E, Otten L. 1989. Molecular systematics of biotype III Ti plasmids of *Agrobacterium tumefaciens*. *Mol. Plant Microbe Interact.* 2:64–74
77. Paulus F, Otten L. 1993. Functional and mutated agrocinopine synthase genes on octopine T-DNAs. *Mol. Plant-Microbe Interact.* 6:393–402
78. Paulus F, Ride M, Otten L. 1989. Distribution of two *Agrobacterium tumefaciens* insertion elements in natural isolates: evidence for stable association between Ti plasmids and their bacterial hosts. *Mol. Gen. Genet.* 219:145–52
79. Pu X, Goodman RN. 1992. Induction of necrogenesis by *Agrobacterium tumefaciens* on grape explants. *Physiol. Mol. Plant Pathol.* 1:241–54
80. Rodriguez P-P, Burr TJ, Collmer A. 1991. Polygalacturonase is a virulence factor in *Agrobacterium tumefaciens* biovar 3. *J. Bacteriol.* 173:6547–52
81. Salomone JY, Crouzet P, De Ruffray P, Otten L. 1996. Characterization and distribution of tartrate utilization genes in the grapevine pathogen *Agrobacterium vitis*. *Mol. Plant-Microbe Interact.* 9:401–8
82. Salomone JY, Szegedi E, Cobanov P, Otten L. 1998. Tartrate utilization genes promote growth of *Agrobacterium* spp. on grapevine. *Mol. Plant-Microbe Interact.* 11:836–38
83. Sawada H, Hiroyuki I, Matsuda I. 1995. PCR detection of Ti and Ri plasmids from phytopathogenic Agrobacterium strains. *Appl. Environ. Microbiol.* 61:828–31
84. Sawada H, Oyaizu H, Matsumoto S, Ieki H. 1992. Differentiation of Agrobacterium taxa based on partial sequences of 16s rRNA. *Ann. Phytopathol. Soc. Jpn.* 58:761–65
85. Schrammeijer B, Hemelaar J, Hooykaas PJJ. 1998. The presence and characterization of a virF gene on *Agrobacterium vitis* Ti plasmids. *Mol. Plant-Microbe Interact.* 11:429–33
86. Schroth MN, McCain AH, Foott JH, Huisman OC. 1988. Reduction in yield and vigor of grapevine caused by crown gall disease. *Plant Dis.* 72:241–46
87. Schulz TF, Lorenz D, Eichhorn KW, Otten L. 1993. Amplification of different marker sequences for identification of *Agrobacterium vitis* strains. *Vitis* 32:179–82
88. Sheng J, Citovsky V. 1996. Agrobacterium-plant cell DNA transport: Have virulence proteins will travel *Plant Cell,* 8:1699–710
89. Staphorst JL, van Zyl FGH, Strijdom BW, Groenewold ZE. 1985. Agrocin-producing pathogenic and nonpathogenic biotype-3 strains of *Agrobacterium tumefaciens* active against biotype-3 pathogens. *Curr. Microbiol.* 12:45–52
90. Stover EW, Swartz HJ, Burr TJ. 1997. Crown gall formation in a diverse collection of *Vitis* genotypes inoculated with *Agrobacterium vitis*. *Am. J. Enol. Vitic.* 48:26–32
91. Stover EW, Swartz HJ, Burr TJ. 1997. Endophytic Agrobacterium in crown gall-resistant and -susceptible *Vitis* genotypes. *Vitis* 36:21–26
92. Süle S. 1978. Biotypes of *Agrobacterium tumefaciens* in Hungary. *J. Appl. Bacteriol.* 44:207–13
93. Süle S. 1986. Survival of *Agrobacterium tumefaciens* in Berlandieri X Riparia grapevine rootstock. *Acta Phytopathol. Acad. Sci. Hung.* 21:203–6
94. Süle S, Burr TJ. 1998. The effect of resistance of rootstocks to crown gall (*Agrobacterium* spp.) on the susceptibility of scions in grape vine cultivars. *Plant Pathol.* 47: 84–88
95. Süle S, Mozsar J, Burr TJ. 1994. Crown gall resistance of *Vitis* spp. and grapevine rootstocks. *Phytopathology* 84:607–11
96. Szegedi E, Czako M, Otten L. 1996. Further evidence that the vitopine-type pTi's of *Agrobacterium vitis* represent a novel group of Ti plasmids. *Mol. Plant-Microbe Interact.* 9:139–43
97. Szegedi E, Kozma P. 1984. Studies on inheritance of resistance to crown gall disease of grapevine. *Vitis* 23:121–26
98. Szegedi E, Nemeth J. 1996. Investigation of grape shoots for *Agrobacterium vitis*. *Novenyvedelem,* 32:605–9
99. Szegedi E, Otten L, Czako M. 1992. Diverse types of tartrate plasmids in *Agrobacterium tumefaciens* biotype III strains. *Mol. Plant-Microbe Interact.* 5:435–38
100. Tarbah FA, Goodman RN. 1985. Rapid detection of *Agrobacterium tumefaciens* in grapevine propagating material and the basis for an efficient indexing system. *Plant Dis.* 70:915–20
101. Thomashow MF, Panagopoulos CG, Gordon MP, Nester EW. 1980. Host range of *Agrobacterium tumefaciens* is determined by the Ti plasmid. *Nature* 283:794–96
102. Tinland B, Huss B, Paulus F, Bonnard G, Otten L. 1989. *Agrobacterium tumefaciens* 6b genes are strain-specific and affect the activity of auxin as well as cytokinin genes. *Mol. Gen. Genet.* 219:217–24
103. Turk SCHJ, Nester EW, Hooykaas PJJ. 1993. The virA promoter is a host-range determinant in *Agrobacterium tumefaciens*. *Mol. Microbiol.* 7:719–24
104. Van Nuenen M, De Ruffray P, Otten L. 1993. Rapid divergence of *Agrobacterium vitis* octopine-cucumopine Ti plasmid from a recent common ancestor. *Mol. Gen. Genet.* 240:49–57
105. Wample RL. 1997. Influence of pre- and post-treatment storage on rooting of hot-water-treated cuttings of Cabernet Sauvignon. *Am. J. Enol. Vitic.* 48:131–36
106. Wample RL, Bary A, Burr TJ. 1991. Heat tolerance of dormant *Vitis-Vinifera* cuttings. *Am. J. Enol. Vitic.* 42:67–72
107. Weibgen U, Russa R, Yokota A, Mayer H. 1993. Taxonomic significance of the lipopolysaccharide composition of the three biovars of *Agrobacterium tumefaciens*. *Syst. Appl. Microbiol.* 16:177–82
108. Willems A, Collins MD. 1993. Phylogenetic analysis of rhizobia and agrobacteria based on 16S rRNA gene sequences. *Int. J. Syst. Bacteriol.* 43(2):305–13
109. Yanagi M, Yamasato K. 1993. Phylogenetic analysis of the family Rhizobiaceae and related bacteria by sequencing of 16S rRNA gene using PCR and DNA sequencer. *FEMS Microbiol. Lett.* 107:115–20

110. Yanofsky M, Lowe E, Montoya A, Rubin R, Krul W, et al 1985. Molecular and genetic analysis of factors controlling host range in *Agrobacterium tumefaciens*. *Mol. Gen. Genet.* 201:237–46

111. Zaenen I, Van Larebeke N, Teuchy H, Van Montagu M, Schell J. 1974. Supercoiled circular DNA in crown gall inducing Agrobacterium strains. *J. Mol. Biol.* 86:109–27

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THE THREE DS OF PCR-BASED GENOMIC ANALYSIS OF PHYTOBACTERIA: Diversity, Detection, and Disease Diagnosis

FJ Louws,[1] JLW Rademaker,[2*] and FJ de Bruijn[2,3]

[1]*Department Plant Pathology, North Carolina State University, Raleigh, North Carolina 27695; e-mail, frank_louws@ncsu.edu;* [2]*MSU-DOE Plant Research Laboratory, NSF Center for Microbial Ecology,* [3]*Department of Microbiology, Michigan State University, East Lansing, Michigan 48824;* [*]*Current address: The Netherlands Culture Collection of Bacteria (NCCB), P.O. Box 80056, 3508 TB Utrecht, The Netherlands*

Key Words population structure, polymerase chain reaction, genomic fingerprinting, informatics, disease management, phylogeny

■ **Abstract** The advent of molecular biology in general and the polymerase chain reaction in particular have greatly facilitated genomic analyses of microorganisms, provide enhanced capability to characterize and classify strains, and facilitate research to assess the genetic diversity of populations. The diversity of large populations can be assessed in a relatively efficient manner using rep-PCR-, AFLP-, and AP-PCR/RAPD-based genomic fingerprinting methods, especially when combined with computer-assisted pattern analysis. Genetic diversity maps provide a framework to understand the taxonomy, population structure, and dynamics of phytobacteria and provide a high-resolution framework to devise sensitive, specific, and rapid methods for pathogen detection, plant disease diagnosis, as well as management of disease risk. A variety of PCR-based fingerprinting protocols such as rDNA-based PCR, ITS-PCR, ARDRA, T-RFLPs, and tRNA-PCR have been devised, and numerous innovative approaches using specific primers have been adopted to enhance both the detection and identification of phytobacteria. PCR-based protocols, combined with computer-based analysis, have provided novel fundamental knowledge of the ecology and population dynamics of bacterial pathogens, and present exciting new opportunities for basic and applied studies in plant pathology.

INTRODUCTION

The advent of molecular biology has caused a significant shift in the types of approaches used to characterize and identify plant pathogens and to devise disease management strategies. This shift is driven by both technology and ecology, and has occurred in parallel with significant changes in agricultural production methods, as highlighted by advances in precision agriculture and ecologically based pest

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